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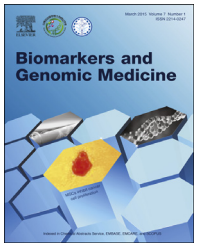

ORIGINAL ARTICLE

Associations of positive epidermal growth factor receptor expression and *K-RAS* gene mutations with various clinicopathological parameters and survival of colorectal carcinoma patients

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Abstract Epidermal growth factor receptor (EGFR) is a member of the transmembrane receptor tyrosine kinase family. In normal and malignant cells, activation of the EGFR cascade is involved in the regulation of various cellular activities. The objective of this study was to identify and assess associations of positive EGFR expression and *K-RAS* mutations with various clinicopathological parameters and survival of colorectal carcinoma patients. EGFR of colorectal cancer (CRC) tissue specimens was subjected to immunohistochemical analysis, polymerase chain reaction, and DNA sequencing. Immunohistochemical staining was performed using monoclonal antibodies against EGFR antigens and examination of mutations was performed to detect mutations in codons 12 and 13 of the *K-RAS*. Statistical analysis was performed using SPSS version 16.0. The results of this study showed that of the 40 study participants, 62.5% (25/40) showed positive EGFR overexpression. Of the patients showing positive EGFR expressions, 52% had mutations in the *K-RAS*. Mutations were spread in codon 12 (64.3%) and codon 13 (35.7%) and there was one sample with mutations in codons 12 and 13

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at the same time. A statistically significant association was found between the presence of metastasis and EGFR overexpression and survival of CRC patients. In addition, a significant association was found between *K-RAS* mutations and metastasis and survival of CRC patients. In conclusion, EGFR overexpression and *K-RAS* mutations were found in CRC patients. Both factors are known to be associated with poor prognosis of cancer patients in terms of patient survival. Early detection of *K-RAS* mutations in CRC patients is a crucial component in the determination of the type of therapy and treatment for the patient.

Introduction

One of the most promising molecular targets is the epidermal growth factor receptor (EGFR), often referred to as HER1. EGFR is a member of the transmembrane receptor tyrosine kinase family. In normal and malignant cells, activation of EGFR receptor cascade is involved in the regulation of various cellular activities, including cell growth, differentiation, and proliferation. The EGFR signaling pathway can also increase cancer cell transformation, angiogenesis, and metastasis.[1] These effects are mediated by multiple signaling mechanisms, such as the signaling pathways of mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase.[2] The high expression of EGFR is known to be associated with exacerbation of tumor stage, treatment resistance[3,4] and, in some types of tumors, poor prognosis.[5,6]

K-RAS is a signal transduction downstream of receptor tyrosine kinases, one of which is EGFR. EGFR signaling pathways are activated when it binds to its ligands, such as the transforming growth factor-α and EGF. Upon EGFR stimulation, K-RAS proteins are immediately activated, leading to a strictly regulated activity of RAF/mitogen-activated protein kinase (MAPK)/extracellular-signal regulated kinase (ERK) signaling pathways. Mutations in the *K-RAS* gene lead to altered activity of the protein, which occur in approximately 30—50% of colorectal carcinoma (CRC) patients.[7] K-RAS protein, also called p21, is a member of the Ras superfamily of proteins, is located on human chromosome 12 and encoded by 189 amino acids, and contains four coding exons and a 5′-noncoding exon.[8]

In normal physiological conditions, the EGFR upstream signals such as EGF can activate K-RAS through the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP). This process occurs only temporarily due to the presence of the GTPase-activating proteins (GAP) that will hydrolyze GTP to GDP again. However, this process will change when *K-RAS* undergoes mutation, resulting in abnormal activity of the GTPase intrinsic to K-RAS, and preventing GAP from hydrolyzing GTP in K-RAS. This leads the K-RAS proteins to accumulate in a GTP-bound form and become permanently activated. Mutant *K-RAS* leads to K-RAS being continuously activated and activates a variety of intracellular signaling pathways.[9,10] Thus, *K-RAS* mutations play an important role in human tumorigenic processes, commonly occurring in patients with pancreatic, thyroid, lung, and colorectal cancer.

Association of EGFR and *K-RAS* mutations with prognosis of cancer patients remains controversial. In Indonesia, there is still a dearth of information on the association of EGFR expression and *K-RAS* mutations with clinical outcomes and survival of CRC patients. Thus, the purpose of the present study was to determine the association of EGFR expression and metastasis with the survival of CRC patients and to analyze association of EGFR expression and *K-RAS* mutations with the survival of cancer patients.

Materials and methods

Patients

The participants were 40 CRC patients treated at the Department of Surgery of Hasanuddin University's Faculty of Medicine/Dr. Wahidin Sudirohusodo Hospital and networking teaching hospitals in Makassar, Indonesia. The study was conducted from June 2010 to October 2010. Patient data collected included: identity (name, age, and sex); location of tumor; metastases; histopathological diagnosis; and grading of differentiation, recurrence, and mortality observed for 1 year after sampling. Paraffin-embedded CRC tissue blocks were collected for further analysis. The study was approved by the biomedical research ethics committee at the Faculty of Medicine of Hasanuddin University with Registration Number UH.10110207 of 2010 and recommendation of ethical approval number 0018/H 04845.31/0036/Komotik/2011.

Hematoxylin—eosin staining

Histopathological profiles of CRC tissue were analyzed using hematoxylin—eosin staining. Sample deparaffinization was performed using xylene and dehydration using alcohol. Slides were then washed with running water for 15 minutes and soaked in hematoxylin solution for 10—15 minutes. Slides were then washed with running water for 15 minutes and dipped in 1% acid alcohol. Subsequently, slides were dipped in liquid ammonia and stained with 1% eosin for 10—15 minutes. Results of staining were observed under a microscope at 400× magnification.

Immunohistochemical analysis of EGFR expression

Immunohistochemical staining was conducted to observe expression of EGFR proteins in CRC tissues. Slides were

deparaffinized using xylene and dehydrated using an alcohol series. Subsequently, slides were soaked in citrate buffer at a pH of 6 and heated at 95°C. After blocking with 3% H_2O_2 in methanol (endogenous blocking), an Ab-5, clone H11, monoclonal antibody (Lab Vision Corp., Freemont, CA, USA) in 0.2% BSA was added. Counterstaining was performed using Mayer's hematoxylin for 5—10 minutes at room temperature. Slides were then Entellan-mounted and observed under a microscope at 400× magnification to count cells expressing EGFR. Positive cells were calculated per 100 cells on at least 10 fields of each slide. EGFR expression was considered positive if it was found in > 1% of tumor cells in partially or completely stained membranes. It was considered negative if ≤ 1% of neoplastic cells showed the specific staining.

DNA extraction of CRC tissues

DNA was extracted from 5—10 sections of 20 μm-thick formalin-fixed, paraffin-embedded cancer tissues using QIAamp FFPE Tissue kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions.

K-RAS amplification

Polymerase chain reaction (PCR) was performed using ABI 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with a volume of 50 μL containing 20mM Tris-HCl pH 8.4, 50mM KCl, 1.5mM MgCl, 200μM dNTP, 400nM sense and antisense primers, 2 units of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), and 5 μL extracted DNA. Primer pairs for the first PCR reaction were 5′-TCATTATTTTTATTATAAGGCCTGCTGAA-3′ and 5′-ATATGCATATTAAAACAAGATTTACC-3′ with a targeted product size of 189 bp. Primer pairs for the second reaction were 5′-GCCTGCTGAAAATGACTGAAT-3′ and 5′-CAAAGACTGGTCCTG CACCAGTA-3′ with a product size of 170 bp. PCR process was performed for 35 cycles with predenaturation and denaturation at 94°C, annealing at 53°C, and elongation and final elongation at 72°C. The second reaction used the same conditions, except for annealing at 55°C and for template that used 5 μL of PCR product of the first reaction. Successful PCR was confirmed by agarose gel electrophoresis at 80 V for 30 minutes. Positive results were marked by 189-bp DNA band from the first PCR reaction or 170-bp DNA band from the second PCR reaction.

DNA fragment purification

Purification of DNA fragments from PCR reactions was performed using QIAQuick kit (Qiagen). Purification was carried out by gel cut purification for PCR products containing nonspecific bands.

DNA sequencing

Sequencing began with sequencing cycle on 15 μL 1× buffer solution containing 1 unit of BigDye terminator version 3.1 (Applied Biosystems), 0.2μM primers, and 2—5 μL of DNA fragments of purified PCR products. The sequencing cycle reaction was run at denaturation temperature of 96°C for 10 seconds, annealing temperature of 50°C for 5 seconds, and elongation temperature of 60°C for 4 minutes, for 25 cycles. Predenaturation was carried out in the beginning of the reaction at 96°C for 3 minutes. Sequencing was performed using capillary-based ABI 3130xl DNA sequencer (Applied Biosystems). The dried sample was dissolved by adding 11 μL of Hidi solution, and then put in a 96-well plate. DNA sequencing electropherogram was downloaded for editing and analysis. Editing was done using BioEdit version 7.0.5 (Ibis Bioscience, Carlsbad, CA, USA) and FinchTV version 1.4.0 (Geospiza, Inc. Seattle, WA, USA). In order to determine the occurrence of mutations edited sequencing results were aligned with *K-RAS* sequences downloaded from GenBank with Access Number NC_000012: c25295121-25249447.

Statistical analysis

Data were processed and then analyzed descriptively using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA) in order to determine the association between EGFR overexpression and *K-RAS* mutations with metastasis/recurrence and patient mortality. Then, data were statistically tested using Pearson's Chi-square test. Overall survival rates were calculated by the Kaplan—Meier method, and the differences in survival rates were analyzed by the log-rank test. Significance was determined at $p < 0.05$. If data were unqualified for Pearson's Chi-square test, Fisher's exact test was used.

Results

Patients and clinical characteristics

Table 1 summarizes the overall patient and clinical characteristics. Participants of this study consisted of 57.5% men and 42.5% women aged 15—71 years (mean ± standard deviation = 52.82 ± 13.57 years). Age was < 40 years in 22.5% and ≥ 40 years in 77.5%. The highest percentage of the participants (55.0%) was of the Bugis ethnic group, followed by Makassar (27.5%), Toraja (12.5%), and Javanese (5.0%).

Based on CRC sites, 57.5% were found in the rectum, 25.0% in the sigmoid colon, 10.0% in the ascending colon, 5.0% in the transverse colon, and 2.5% in the descending colon. A histopathologically low grade was found in 55.0% of patients and a histopathologically high grade in 45.0%. Immunohistochemical examination of 40 tumor tissue samples found 62.5% (25/40) of patients with positive EGFR expression and 37.5% (15/40) with negative EGFR expression (1% cutoff point).

Association of EGFR overexpression with histopathological grading of CRC patients

Analysis of the association of EGFR overexpression with histopathological grading of CRC patients indicated that, of 25 patients with positive EGFR expression, 13 (32.5%) showed a histopathologically low grade and 12 (30.0%) showed a high histopathologically grade. In patients with

Table 1 The distribution of patients and clinical characteristics.

Characteristics	Distribution	
	No. (n = 40)	%
Sex		
Male	23	57.5
Female	17	42.5
Age		
15–40 y	9	22.5
41–80 y	31	77.5
Race		
Bugis	22	55.0
Makassar	11	27.5
Toraja	5	12.5
Javanese	2	5.0
Tumor location		
Ascendens colon	4	10.0
Transversum colon	2	5.0
Descendens colon	1	2.5
Sigmoid colon	10	25.0
Rectum	23	57.5
Pathologic diagnosis		
Adenocarcinoma	33	82.5
Mucinous adenocarcinoma	7	17.5
Histopathology grading		
Low grade	22	55.0
High grade	18	45.0

negative EGFR overexpression, we found nine (22.5%) with histopathologically low grade and six (15.0%) patients showed a high histopathologically grade, as seen in Table 2.

Association of EGFR overexpression with metastasis of CRC patients

Table 3 shows the association of EGFR overexpression with metastasis of CRC patients. Of 62.5% (25/40) of positive samples with EGFR overexpression, 40.0% (16/40) of patients had metastasis. Of those with negative EGFR overexpression, 10% (4/40) of patients had metastasis. A significant association (p = 0.022) of metastatic incidence with EGFR overexpression was found.

Survival of CRC patients based on EGFR overexpression

CRC patients with positive EGFR overexpression had a survival of 15.4 months and 100.0% for up to 6 months. After 8–10 months, survival declined to 90.0%. After 10–11 months, survival declined to 86.0%, and subsequently to 76.0% after 17 months. CRC patients with negative EGFR overexpression had a survival of 17.0 months and 100.0% for up to 17 months. After 17 months, survival declined to 76.0% (Fig. 1).

Table 2 The association between EGFR overexpression and histopathology grading.

EGFR overexpression	Histopathology grading		Total (n = 40)
	Low grade (n = 22)	High grade (n = 18)	
Negative	9	6	15
Positive	13	12	25

Table 3 The association between EGFR overexpression and metastasis incidence.

EGFR overexpression	Metastasis incidence		Total (n = 40)
	Present (n = 20)	None (n = 20)	
Negative	11	4	15
Positive	9	16	25

Survival of CRC patients based on EGFR overexpression and K-RAS gene

CRC patients with positive EGFR expression or CRC patients with *K-RAS* mutation had a survival rate of 16.3 months. CRC patients with positive EGFR overexpression and *K-RAS* mutation had a lower survival rate of 14.0 months. CRC patients with negative EGFR overexpression and *K-RAS* normal had survival rate of 17.0 months.

CRC patients with positive EGFR overexpression or *K-RAS* mutation had a 100.0% survival up to 9 months, after which it declined to 90.0%. CRC patients with positive EGFR overexpression and *K-RAS* mutations obtained had a 100.0% survival up to 6 months, which it dropped to 80.0% after 6–10 months, and dropped again at 11 months to 64.0% (Fig. 1). CRC patients with negative EGFR overexpression and normal *K-RAS* had a 100.0% survival up to 17 months, after which it declined to 74.0%.

Discussion

Results show that CRC was most often found in the rectum (57.5%). This differs from previous studies that found that CRC was most often found in the colon, with rectum as the second most frequent site of CRC.[11,12] EGFR overexpression and histopathological grading in this study showed no significant association (p = 0.512). This result was supported by other studies that reported no association of histopathological grading with EGFR overexpression.[13] Immunohistochemical analysis of EGFR expression in this study indicated that 62.5% of the samples had positive EGFR expression.

EGFR protein, often referred to as HER1, is a member of the transmembrane receptor tyrosine kinase family, in which cascade activation of this receptor is involved in the regulation of cell growth, proliferation, and differentiation. The EGFR signaling pathway can also enhance malignant transformation, angiogenesis, and metastasis.[1] EGFR catalyzes the transfer of phosphate molecules from ATP to active sites of the tyrosine kinase to mediate the signaling cascade. Upon binding to its ligand, EGFR will undergo homodimerization and transphosphorylation of several tyrosine kinase domains to initiate intracellular EGFR signaling pathways, which, among others, include

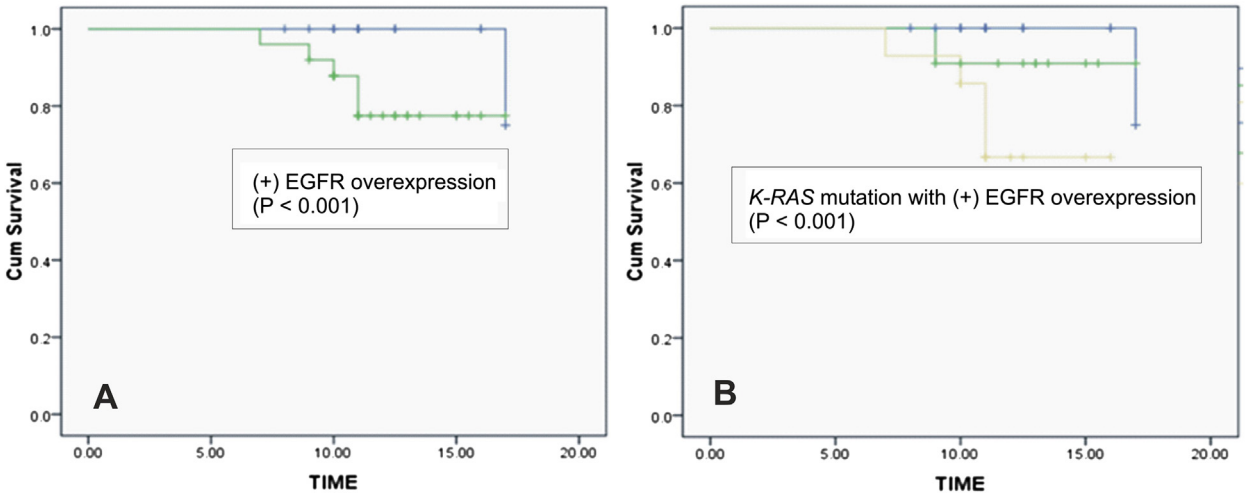

Figure 1 The Kaplan–Meier survival curve for colorectal carcinoma. (A) The blue line is negative EGFR. The green line is positive EGFR. (B) The blue line is wild type *K-RAS* and negative EGFR. The green line is *K-RAS* mutation or positive EGRF. The brown line is *K-RAS* mutation and positive EGFR.

activation of signal transducer dan activator of transcription (STAT) proteins, Sarcoma-family kinases (SRC) family kinase, Protein kinase B (AKT) proteins, and MAPK signaling pathways. Activation of those various proteins and signaling pathways will subsequently induce transcription of genes involved in various cellular processes such as cell division and self-defense, all of which describe the role of EGFR in the process of tumor cell growth.[14]

EGFR overexpression has been reported in 25–82% of patients with colorectal cancer[15]; however, its clinical significance in colorectal cancer remains unclear. A study conducted in 249 colorectal cancer patients showed an association of EGFR overexpression with tumor grade (low differentiation; $p = 0.014$),[16] but another study showed no such association.[17] In addition, there were other studies that found an association of EGFR overexpression with decreased patient survival rate,[13,17] but other studies found no such association.[15]

The present study found a significant association ($p = 0.050$) of EGFR overexpression with patients' clinical outcome of metastatic process. Other studies have shown that 50% of CRC patients had metastases that contributed to the high rate of mortality in CRC patients. EGFR expression was reported to be associated with increased aggressiveness of the disease,[18] increased risk of metastasis,[19] and a more severe stage of the tumor.[20] Several studies have shown that metastatic lesions were present in the genomic differences of the primary tumor.[21,22] A previous study showed that a higher EGFR reactivity is found in lymph nodes and metastases compared to primary tumors.[13] In addition, EGFR reactivity in the innermost region of the primary tumor correlates most strongly with EGFR reactivity in the lymph nodes and metastatic liver. Patients with positive EGFR overexpression showed a poorer prognosis compared with those with negative EGFR overexpression, and showed a significant correlation with advanced stages of cancer. A study in Taiwan concluded that EGFR expression has a prognostic value only for patients with metachronous, and not synchronous, metastasis.[23]

EGFR-positive patients with mutated *K-RAS* gene had a significantly lower rate of survival than those with no *K-RAS* mutations. PCR analysis indicated that 52% of EGFR-positive patients had *K-RAS* mutation. All patients with *K-RAS* mutation were known to have metastatic conditions. Mutations in the *K-RAS* are thought to cause a less properly regulated increase in EGFR signaling pathways. Data of the present study showed that *K-RAS* mutations could be used as a marker of aggressive tumor phenotype, as shown by the association of *K-RAS* mutation with metastatic cancer cells. However, the prognostic significance of *K-RAS* mutation remains controversial. Incidence of mutations in *K-RAS* in several studies was related to the shortening of patient survival.[24–26] However, other studies found no correlation of *K-RAS* mutation with patients' survival rate.[27–29] A previous study showed an increase in the mortality rate of colon cancer patients with a *K-RAS* mutation in codon 13 (G → A), but not *K-RAS* mutations in general.[24]

In addition, *K-RAS* mutations do not have a prognostic value in patients with metachronous or synchronous metastasis of CRC.[30] This raises a speculation that the prognosis of cancer patients is associated with specific mutations of *K-RAS* and may be related to the race and environmental factors among patients.

There are three RAS in the human genome, *K-RAS*, *HRas*, and *NRas*. Mutations of the *K-RAS* have detected in 35–45% of CRC patients,[30–33] whereas NRas and HRas mutations were only found in 1–3% of patients with CRC. In the present study, 64.3% of *K-RAS* mutations were distributed in codon 12 and 35.7% in codon 13, and there was one patient with mutations in codons 12 and 13. These results are consistent with previous studies,[34] which showed that mutations of *K-RAS* mostly occurred in codon 12 (77%) and codon 13 (20%) in exon 2 of the *K-RAS*. A somatic missense mutation in codon 12 of the *K-RAS* lead to substitution of a single amino acid (Gly → Val), which is the most common abnormality found in CRC.

K-RAS encodes GTPase protein that plays a role in the EGFR-induced downstream cell signaling; thus, this protein

participates in the highly important activation of oncogenic signaling pathways. RAS protein is a key element of the intracellular RAS-MAPK signaling pathway, which leads to the activation of several other signaling pathways that control mechanisms of gene transcription, cell proliferation, apoptosis, angiogenesis, invasion, and migration.[35] RAS proteins that play a role in the process of signal transduction are normally present in the form of active (RAS-GTP) and inactive (RAS-GDP) conformations. RAS proteins are activated by guanine nucleotide exchange factor and inactivated when RAS-GTP is hydrolyzed to RAS-GDP by GAPs. In normal cells, guanine nucleotide exchange factor and GAP activity are strictly regulated. Mutations in *K-RAS* are capable of leading to a decreased intrinsic GTPase activity of RAS proteins so that the proteins become resistant to GAPs. This leads the mutated RAS proteins to be permanently in the active state (RAS-GTP) and continuously activates signaling pathways, despite the lack of induction of cell surface receptors.[36–38] Activation of mutations is thought to be capable of inducing oncogenic transformation and nonsensitivity to anti-EGFR antibody therapy, leading to EGFR-independent activation of phosphatidylinositol-3 kinase/AKT and MAPK signaling pathways.

In conclusion, EGFR overexpression and *K-RAS* mutations were found in CRC patients. Both factors are known to be associated with poor prognosis of cancer patients in terms of patient survival. Early detection of *K-RAS* mutations in CRC patients is thus a crucial component in the determination of the type of therapy and treatment for the patient.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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